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Applicants: Rheins and Morhenn Art Unit: 1646  
Application No.: 09/375,609 Examiner: S. Prasad  
Filed: August 17, 1999

Title: METHODS AND KITS FOR OBTAINING AND ANALYZING SKIN SAMPLES FOR THE DETECTION OF NUCLEIC ACIDS (as amended)



Commissioner of Patents  
Washington, D.C. 20231

DECLARATION OF  
APPLICANTS UNDER 37 C.F.R. § 1.132

Sir:

We, Lawrence A. Rheins and Vera B Morhenn, co-inventors of the above-identified application, do hereby declare and state that:

1. We are familiar with the above-identified patent application and the disclosure in the Specification of methods for obtaining and analyzing skin samples.
2. We understand that claims 64 to 136 have been rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement.
3. We, or others under our direction, have performed experiments using methods disclosed in the above-identified application to obtain by a non-invasive method, a skin sample for detecting nucleic acids in the skin sample.
4. The experiments show that different types of nucleic acids can be detected or isolated from skin samples. Nucleic acids detected included interleukins (IL) such as IL-1, IL-4, IL-8 and Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ), and housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GADPH). Our experimental protocol and the results obtained are described in detail in Exhibit A, attached hereto.

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5. We further declare that all statements made herein of knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

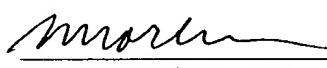
10/30/01

Date

  
Lawrence A. Rheins, Ph.D.

11/2/01

Date

  
Vera B Morhenn, M.D.

Attachment: Exhibit A

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**EXHIBIT TO DECLARATION**  
**TAPE STRIP STUDY FOR HOUSEKEEPING GENES AND CYTOKINES**

**INTRODUCTION**

Skin samples for the detection or isolation of nucleic acids can be obtained using a non-invasive method. The method includes applying tape strips to skin to remove skin cells followed by isolation or detection of nucleic acids in the skin sample.

A nucleic acid profile obtained from a skin sample can be diagnostic of a variety of conditions. For example, following an allergic response, affected skin can show a nucleic acid profile having increased cytokines as compared to skin prior to the allergic response. In addition, nucleic acids encoding proteins that are not specific to skin or a skin response can also be detected in skin samples. The present study shows that a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), can be detected in a skin sample obtained by non-invasive tape-stripping methods.

**MATERIALS AND METHODS**

**Sample Preparation:** Patches containing either irritant (1% sodium lauryl sulfate (SLS) in distilled water) or vehicle (distilled water) was applied to skin sites on the upper back of each subject. Control sites were upper back skin sites having a normal appearance. For each treatment (irritant, vehicle, none), three sites were tested. Aqueous treatments were applied to Webril patches with a pipette, and the patches were affixed to the upper back. After 24 hours, the patches were removed and each site was tape stripped

**Tape stripping procedure:** Each test site was tape stripped 10 times with individual, 22 mm D-SQUAME® tape strips (CuDerm, Dallas, TX). Tape stripping was performed using gloved hands, and applying firm, even pressure to the tape strip over the skin site by rubbing with the fingertips in a circular motion (3 revolutions). Each tape strip was rapidly removed from the skin with the fingertips using the tabbed area, and placed in a sterile, RNase free microcentrifuge tube for immediate extraction.

**Pooling of tape strips:** Sequentially applied tape strips were combined in pools of five for sequential extraction followed by isolation of RNA. Thus, tape strips 1-5 and 6-10 from a given site created two distinct samples for separate analysis.

**Extraction of tape strips:** Each tape strip was individually extracted in lysis buffer (Qiagen RLT buffer containing β-mercaptoethanol) by a vortex and incubation method (vortex sample for 30 seconds, incubate 1 minute, vortex 30 seconds), and the sample was spun in a microcentrifuge for 10 seconds. The tape was removed from the tube using sterile tweezers, and discarded. The next tape was then applied to the skin site, removed rapidly, placed in the microcentrifuge tube containing the lysis buffer from the prior tape extraction and extracted as described. The tape stripping and extraction procedure was repeated to form an extract from five pooled tape strips. One sample blank was prepared

for each subject consisting of five tapes, generated (taken out of the box) at the end of the tape stripping procedure, and extracted as described.

**Isolation of Total RNA** Total mRNA was isolated at Althea Technologies using the Qiagen RNeasy purification kit.

**Quantitative Reverse Transcriptase Polymerase Chain Reaction (Q-RT-PCR)**  
Quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR) was performed using Taqman technology by Althea Technologies, Inc., San Diego, CA. mRNA encoding the following proteins was assessed: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-8 (IL-8) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). The housekeeping gene, GAPDH, was used as a control for each sample and to normalize the data. Additionally, for reference, commercially available placental total RNA (18 nanograms) was used as a positive control and reference.

## RESULTS

Total RNA in each pool was analyzed for the following mRNAs: IL-1; IL-4; IL-8; TNF $\alpha$ ; and GAPDH using semi-quantitative PCR. Results are summarized below and shown in Table 1.

- a. mRNAs encoding GAPDH and the cytokines IL-1, IL-8, TNF- $\alpha$  can be detected.
- b. mRNA can be obtained from and detected in skin that is exposed to an irritant as well as in skin that is exposed to vehicle alone and in uninvolved skin.
- c. The first five tape strips (forming nucleic acid extract of Pool A) are sufficient for detection of mRNAs encoding GAPDH, IL-1, IL-8 and TNF- $\alpha$ .
- d. mRNA for IL-4 could not be detected in any samples including the control RNA.

In Table 1, the value, Ct, is the number of PCR cycles required to produce a standard reference fluorescence value (the number of cycles required to produce a standard number of molecules of PCR product). Values above 40 indicate that mRNA is undetectable. Each value presented is an average of the values obtained for the three sites having the same treatment (irritant, vehicle, none).

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Table 1

<u>Subject 1</u>	<b>GAPDH</b>	<b>IL-1</b>	<b>IL-4</b>	<b>IL-8</b>	<b>TNF<math>\alpha</math></b>
Irritant, Pool A	33.22	34.06	45.00	28.43	36.47
Irritant, Pool B	31.57	30.30	45.00	28.29	35.65
Vehicle, Pool A	32.31	38.86	45.00	30.59	39.32
Vehicle, Pool B	30.92	32.32	42.65	28.41	34.25
None, Pool A	33.94	45.00	45.00	35.25	43.14
None, Pool B	33.54	35.66	45.00	32.77	40.01
<u>Subject 2</u>					
Irritant, Pool A	28.96	34.23	40.74	28.23	36.53
Irritant, Pool B	28.96	36.93	42.77	29.77	34.09
Vehicle, Pool A	28.94	32.64	39.31	25.81	37.08
Vehicle, Pool B	28.23	30.10	38.59	27.31	34.24
None, Pool A	34.14	45.00	45.00	36.89	44.00
None, Pool B	34.95	45.00	45.00	34.16	43.31
Placental RNA	22.53	27.68	45.00	26.33	30.61
Blank Tape	42.28	45.00	45.00	37.53	43.95

### CONCLUSIONS

The data demonstrate that mRNAs encoding various proteins can be detected in skin samples obtained using a non-invasive tape stripping method. mRNA encoding the cytokines IL-1, IL-8 and TNF- $\alpha$  and the housekeeping gene, GAPDH, can be detected and quantified in skin samples.

The data also reveal that the first five tape strips contain sufficient sample for the detection of mRNAs. Thus, the method used to obtain skin samples is non-invasive.